Manipulating EBV plasmids II: Linear Transformation

Modified by Jisook Jan/04

Transformation of linear fragments

Background

The prerequisite for this method is that you have at hand a construct with a selectable marker (usually an antibiotic resistance gene) interrupting (if its a k.o.) or close to the manipulated gene. Recombination frequencies usually are good starting from approx. 1 kB of homologous sequence on each side. The plasmid carrying this construct is then restricted so that the vector backbone is cleaved at multiple sites, which makes gel purification of the fragment to be transformed unnecessary.

In the classical method, linear DNA is transformed into *E. coli* BJ5183 carrying the EBV genome (usually, p2089). This strain is recA+ (i.e. able to do homologous recombination) and recBC- (i.e. unable to degrade linear DNA fragments), so the protocol is quite straightforward. However, EBV plasmids are only moderately stable in this recA+ strain and yields in DNA preparations are not satisfactory. For these reasons, constructs have to be transferred into DH10B, a recA- strain. These transfers of 180 kB constructs between *E. coli* strains can be difficult and sometimes grow out to be constant sources of frustration.

To circumvent this, we constructed a plasmid (p2650) (pKD46-p3234 in Bill's lab; http://haema145.gsf.de/ham/2650.html) with a temperature sensitive origin of replication containing all functions necessary to allow linear transformation in DH10B.

Permissive temperature : 30ºC, nonpermissive temperature : 42ºC

protocol

Day 1: transformation of p2650(pKD46 which is p3234 in Bill's lab)

p2650(pKD46, p3234 in Bill's lab) is first transformed into *E. coli* DH10B containing the EBV plasmid to be manipulated (usually, this will be p2089). This can be accomplished by any standard transformation method that seems suitable, e.g. the "TSS" method (http://haema145.gsf.de/ham/tsstra.html). Cells should be plated on Agar containing 30ug/ml Chloramphenicol (in the case of p2089) + 30ug/ml Ampicilline (pKD46 is AmpR)

Grow o/n at **30ºC**.

<TSS method >

- grow up E.coli (2089) until O.D. =0.4
- take 1ml of O.D.=0.4 E.coli and spin it down for 1min at max speed
- resuspend pellet in 100ul TSS
- add 5ul (0.5ug DNA) pKD46 (RecA plasmid that has temperature sensitive mutant)
- incubate 30min on ice
- add 1ml LB and incubate 1hr at 30ºC
- Plate on LB+ CAM+Amp plate

Day 2: preparations and preculture to make electrocompetent cells

For linear fragment mutagenesis the plasmid pKD46 has to be introduced into DH10B containing the viral F plasmid. In addition, these bacterial cells have to be prepared for electroporation and the expression of the recombination functions rec α,β,γ is induced by addition of L-arabinose to the growth medium. All preparation steps should be done as cold as possible and permantly on ice. All used material should be precooled to at least 0ºC, including the rotor for centrifugation. Precool the 5ml glass pipettes before use by pipetting cold 10% glycerol up and down several times.

Prepare the following:

- 250 ml sterile water (put in the fridge overnight)
- 500 ml 10% glycerol (sterile) (put in the fridge overnight)
- sterile 500 ml centrifugation bottles in fridge o/n
- sterile 25ml pipet, e-tube in freezer for o/n
- make fresh as required a sterile 10%(w/v) stock solution of L-arabinose (Sigma Aldrich,Inc #A3256) in standard LB medium

In the evening: make a 50 ml o/n culture containing the appropriate antibiotic(s) – Cam and Amp, grow at **30ºC !**

Day 3: electrocompetent cells and electroporation

- inoculate the 25ml o/n culture into 1L of medium containing CAM, Amp and 0.1%(w/v) L-arabinose
- grow at **30ºC** to an OD600nm of approx. 0.3-0.5
- put on ice for 15 min
- harvest cells by centrifugation (4ºC, 10 min, 7000 rpm if it is a GSA rotor, use 2 500ml bottles) remove supernatant
- resuspend cells into 150 ml cold 10% glycerol
- centrifuge at 4ºC, 7000 rpm, 10 min , remove supernatant
- resuspend in 100 ml 0ºC 10% glycerol, centrifuge as above
- resuspend with 2ml 10% glycerol, aliquot 110 µl each into cold e-tubes -> use immediately (or freeze and store at -70ºC)

electroporation:

- use 50 µl of cells and approx. 0.5 - 1 µg of fragment
- electroporation conditions are: 2 mm cuvettes, 2500 V, 25 µFd, 200 Ohms
- after the pulse, resuspend cells in 2 - 3 ml LB/CAM
- grow at 30ºC for 2 - 3 h
- plate on LB/chloramphenicol + the antibiotic selecting for your fragment
- grow at 42ºC o/n (to remove p2650)
- also plate out a control for your linear fragment to see if it is completely digested

Day 4: Purification

in the morning you should have colonies on the plate, which should be purified at least once on double-selection plates; after this, single selection for chloramphenicol usually is sufficient. (double-selection plates are more safe) Once purified, the clones can be characterised, e. g. by restriction analysis (http://haema145.gsf.de/ham/minlys.html).

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